MULTI-ELEMENT SPECTROMETRIC ANALYSIS OF STINGLESS BEE HONEY IN JOHOR FOR FORENSIC PROVENANCING APPLICATION

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This research is dedicated to my lovely wife, Siti Sariyana Bt. Mohd Sapian who has been my constant source of inspiration and to my mother Hambariah Hj. Omar, my late father Shadan Bin Mat Isa (*Al – Fatihah*) and finally, to all my lovely kids.

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ABSTRACT

Consumption of stingless bee honey as nutritious food supplement has been gaining popularity in many countries including Malaysia. However, its safe consumption following the possible presence of toxic amounts of trace elements remains unclear. Hence, this cross-sectional research that assessed the concentrations of multi-elements in purely harvested stingless bee honeys from bee keepers at four geographical locations (five districts) in Johor using inductively coupled plasma-optical emission spectrometry deserves consideration. Even though the concentrations of As, Pb, Cd and Sb in all the purely harvested stingless bee honeys complied fully with the Malaysian Food Standard, however the Cd concentrations in samples from Kota Tinggi was found to be higher than the suggested value by the US Food and Drug Administration for food supplements. While the concentrations of Cu and Cr in pure stingless bee honey samples from Muar and Kota Tinggi alone exceeded the minimal risk levels (MRLs) for chronic oral exposure, higher concentrations than the prescribed MRL for chronic oral exposure of Se for long term effect as set by US Agency for Toxic Substances and Disease Registry (ATSDR) was found in all the pure samples. Except for Ba and Sn, significant variations ($p < 0.05$) in the concentrations of other elements in pure stingless bee honey samples collected from the five districts of Johor were observed. Statistical approach using Principle Component Analysis (PCA) demonstrated 87.0% correct classification and the classification improved to 96.2% with the use of Linear Discriminant Analysis (LDA). This indicates that discrimination was possible for the different geographical regions. Therefore, in view of minimizing threats towards public health and promoting better international acceptance for Malaysian stingless bee honeys, adoption of more stringent maximum permissible limits may prove necessary. Hence, utilization of multi-elements analysis coupled with chemometrics techniques for assigning the provenance of stingless bee honeys for forensic applications is supported.

ABSTRAK

Pengambilan madu kelulut sebagai makanan tambahan berkhasiat semakin dikenali di serata dunia termasuk di Malaysia. Walau bagaimanapun, pengambilan selamat madu kelulut berikutan kehadiran amaun toksik logam surih masih tidak jelas. Oleh itu kajian keratan rentas semasa penentuan kepekatan pelbagai logam di dalam madu kelulut tulen yang diperoleh daripada penternak madu di empat lokasi geografi (lima daerah) di Johor menggunakan plasma gandingan aruhan-spektrometri pancaran optik adalah wajar. Walaupun kepekatan As, Pb, Cd dan Sb di dalam kesemua sampel madu kelulut tulen mematuhi Piawaian Makanan Malaysia, namun kepekatan Cd di dalam sampel dari Kota Tinggi didapati lebih tinggi daripada nilai cadangan oleh pihak *US Food and Drug Administration* untuk makanan tambahan. Manakala kepekatan logam Cu dan Cr di dalam sampel madu kelulut tulen dari Muar dan Kota Tinggi melebihi aras risiko minimum (MRLs) untuk pendedahan oral kronik, kepekatan yang lebih tinggi daripada MRL yang ditetapkan untuk pendedahan kronik oral Se untuk kesan jangka masa panjang seperti ditetapkan oleh pihak *US Agency for Toxic Substances and Disease Registry* (ATSDR) didapati untuk kesemua sampel tulen. Selain daripada Ba dan Sn, variasi signifikan ($p < 0.05$) dicerap dalam kepekatan unsur lain dalam sampel madu kelulut tulen yang dikumpul dari lima daerah di Johor. Pendekatan statistik menggunakan Analisis Komponen Prinsipal (PCA) memberikan 87% ketepatan klasifikasi dan klasifikasi meningkat kepada 96.2% dengan penggunaan Analisis Diskriminasi Linear (LDA). Ini menunjukkan bahawa diskriminasi boleh dibuat berdasarkan kedudukan geografi. Oleh itu, dalam usaha untuk mengurangkan risiko terhadap kesihatan orang awam dan menambahbaik penerimaan madu kelulut Malaysia di peringkat antarabangsa, penggunaan aras maksimum yang dibenarkan yang lebih ketat adalah perlu. Justeru itu penggunaan analisis pelbagai unsur bersama teknik kimometrik dalam menentukan asal madu kelulut bagi tujuan aplikasi forensik adalah didokong.

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LIST OF ABBREVIATIONS

AAS	-	Atomic Absorption Spectrometry
ATSDR	-	Agency for Toxic Substances and Disease Registry
E-SEM	-	Environment Scanning Electron Microscope
FIAS	-	Flow Injection Analysis
GF-AAS	-	Graphite Furnace Atomic Absorption Spectrometry
ICPAES	-	Inductively Coupled Plasma Atomic Emission Spectrometry
ICP-MS	-	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	-	Inductively Coupled Plasma Optical Emission Spectrometry
LDA	-	Linear Discriminant Analysis
PCA	-	Principal Component Analysis
USFDA	-	United State Food and Drug Administration

LIST OF SYMBOLS

Ag	-	Silver
Al	-	Aluminium
As	-	Arsenic
B	-	Boron
Ba	-	Barium
Be	-	Beryllium
Cd	-	Cadmium
Co	-	Cobalt
Cr	-	Chromium
Cu	-	Copper
Fe	-	Iron
Mg	-	Magnesium
Mn	-	Manganese
Mo	-	Molybdenum
Ni	-	Nickel
Pb	-	Lead
Sb	-	Antimony
Se	-	Selenium
Sn	-	Tin
Zn	-	Zinc

CHAPTER 1

INTRODUCTION

1.1 Background of Study

1.1.1 Definitions of Honey

According to Codex Alimentarius (2001), honey is a naturally sweet substance produced by honeybees from nectar plants or from a secretion of living parts of plants, which the bees collect, deposit, dehydrate, store and leave in honeycombs to ripen. This natural product is valuable as it is the only concentrated form of sugar available worldwide and is also used as a food preservative, as mentioned by Aghamirlou *et al.,* (2015).

Honey from different sources may vary in trace element, but generally they all consists the following basic sugar components, such as glucose and fructose. Saba *et al.,* (2013) mentioned that the honey possesses numerous nutrition, healing and prophylactic properties that are suitable for medical treatments (Vit *et al.,* 2015). However, if elements in the honey are above permitted levels, it will pose threats to human body and give negative effects due to contaminants. The contaminants such as Arsenic (As) is likely to come from micro polluting agents in the environment, as mentioned in previous studies by Chandrama *et al.,* (2014).

1.1.2 Uses of Honey

Honey that is produced acts as food store for the bees' colony when there are no flowers and enable the bees to survive through seasons when they are not able to forage because of rain or other adverse circumstances. For human being, honey is a useful source of high carbohydrate food, and usually contains abundant nutrition for human diets. In many countries, honey is regarded as a medicine or special tonic, besides being daily food. It does contain medicinal properties that is acknowledged in modern medicine as a study published in the Pediatric journal that reveal honey as a remedy in helping children with cough at night. Cohen *et al.,* in 2012 concluded that "Honey may be a preferable treatment for cough and sleep difficulty associated with childhood URI (upper respiratory infection)."

Consumption of honey has been gaining considerable popularity as one of the expensive food supplement commodities worldwide (Cohen *et al.,* 2012) owing to its nutritional and medicinal benefits. About 2,500 matrix tonnes of honey was traded in 2013 for Malaysia i.e. accountable for about USD 116 million and USD 23 million worth of import and export, respectively (FAO Statistics Division 2016). This can be attributable to overwhelming empirical evidence advocating for its antimicrobial (Sgariglia *et al.,* 2010), antioxidant (Silva *et al.,* 2013), anti-inflammatory (Ahmad *et al.,* 2012), antihyperlipidemic (Rahman *et al.,* 2016), antidiabetic (Erejuwa *et al.,* 2012) and cardio protective properties (Rao *et al.,* 2016). Moreover, the geographical regions (Rao *et al.,* 2016), climate and environmental conditions as well as the different species of bees (Chua *et al.,* 2012) has been indicated as the determinant factors for the quality of honeys. In Malaysia, honey produced by stingless bees (Trigona species) remains one of the popular choices with its market price ranging from USD 50-100 per kg (Kelly *et al.,* 2014).

1.1.3 Multi-elements Analysis by Inductively Coupled Plasma-Optical Emission Spectrometry

Inductively coupled plasma-optical emission spectrometry (ICP-OES) is one of the most popular analytical tools for determination of elements in various types of samples. ICP-OES is a technique that is more preferred for inorganic element analysis. However, only very few researchers have concentrated on inorganic compounds in honey as mentioned by Mbiri *et al.,* (2011) compared with organic compound. To our best knowledge, there is not much research work performed with regards to multi-elements analysis in stingless bee honey using ICP-OES technique, especially in Malaysia.

Since the chemical properties of honey relies mainly on the absorption of minerals/nutrients through plants from soils (Vanhanen *et al.,* 2011), prolonged contamination by chemical pollutants (e.g. metals and pesticides) from industrial/agricultural seepages into soil and water may possibly lead to serious bioaccumulation concern. Previous researchers have reported about considerable amounts of Cd (1.03 mg/kg), Pb (0.691 mg/kg) and Cu (2.93 mg/kg) in stingless bee honeys sampled from various suppliers throughout Peninsular Malaysia, including 3 samples from Johor Bahru (Moniruzzaman *et al.,* 2014). Alarmingly, the amount of Cd that they reported (Moniruzzaman *et al.,* 2014) exceeded the maximum permitted proportion for honey as prescribed by the Fourteenth Schedule of the Malaysian Food Act 1983 (Act 281) & Regulations (2014). Hence, chronic toxicity of Cd towards kidney, induction of lung tumors as well as skeletal deformities (Flanagan *et al.,* 2008), following the prolonged consumption of contaminated honeys could not be ruled out.

Although the level of Pb (0.691 mg/kg) was reportedly lower than the maximum permitted proportion (2 mg/kg) allowable by the Malaysian law, lower recommended level (0.3 mg/kg) has been suggested for babies, children and the elderly (WHO 2007). This situation renders such honeys as inappropriate for consumption for these vulnerable age groups. Because metal contaminations may occur during honey production, harvesting as well as during packaging and transportation, and since the purity of such stingless bee honey products was not reported by previous researchers, the source of contaminations could not be explicitly ascertained. Interestingly, while excessive exposure of essential trace elements (such as Cu) may lead to several detrimental clinical conditions e.g. gastrointestinal bleeding and heart failure (Gulliver, 1991; Flanagan *et al.,* 2008), specific maximum permitted proportion for various metals (except for As, Pb, Hg, Cd and Sb) in honey has not been prescribed in the Fourteenth Schedule of the Malaysian Food Act 1983 (Act 281) & Regulations (2014).

In this context, it is pertinent to indicate that the regulation of honeys that are sold in the Malaysian market (especially those by small scale sellers) remains scarce, despite the availability of specific guideline for safe consumption of honey in Malaysia. Therefore, the compliance of such honeys to the prevailing standard for human consumption can be questionable. Considering (a) negative health implications following acute and/or chronic exposure of metals (Cope *et al.,* 2004; Flanagan *et al.,* 2008) in stingless bee honey and (b) temporal changes that may have occurred at its harvesting sites over time, continuous assessment on the levels of such elements in the purely harvested stingless bee honeys at beekeepers proves to be imperative. This research assessed the concentrations of various elements in purely harvested stingless bee honeys from beekeepers at four different geographical locations (five districts) in Johor to elucidate its potential threat towards public health and for provenance purpose.

1.1.4 Chemometric Analysis

The discipline of chemometrics is mainly related to the use of statistical computing in chemistry. Matthias Otto mentioned in his published Chemometric book in 2007 that some analytical groups in early 1970s were already working with statistical methods that are nowadays known as chemometrics. He also mentioned about an actual definition of chemometric: 'the chemical discipline that uses mathematical and statistical methods to design or select optimal measurement procedures and experiments, and also to provide maximum chemical information by analyzing chemical data. Kreitals and Watling (2014) indicated that chemical signatures resulted from climatological, geochemical and anthropogenic influences 'are incorporated in the region's geology, soils, water and vegetation; making their way through the food chain to higher level organisms' and vary significantly among the different areas. In this context, identifying multi-elements distribution patterns may prove useful for forensic provenance.

Although the use of genetic methods for population assignment has been suggested (Alacs *et al.,* 2010), and because such methods are 'technique dependent and not all genetic tools provide the same resolution' (Kreitals and Watling, 2014), application of multi-element analysis for provenance establishment may greatly benefit on-going forensic investigations. Coupled with chemometrics techniques, the multi-element analysis approach is especially useful when dealing with populations with low genetic divergence with no distinctive boundaries (Alacs *et al.,* 2010). In this context, the use of chemometrics techniques such as principal component analysis (PCA) and linear discriminant analysis (LDA) has been suggested. While applications of such integrated approach for provenance establishment of coffees (Valentin and Watling, 2013), wines (Martin *et al.,* 2012), pigs (Kreitals and Watling, 2014), beef (Heaton *et al.,* 2008), vegetables (Yan *et al.,* 2015) and honey (Baroni *et al.,* 2009; Chua *et al.,* 2012) have been indicated, specific studies focusing on provenance of stingless bee honeys have not been reported.

1.2 Problem Statements

The regulation of honeys that are sold in the Malaysian market (especially those by small scale sellers) remains limited and the specific maximum permitted proportion for various metals (except for As, Pb, Hg, Cd and Sb) in honey has not been prescribed in the Fourteenth Schedule of the Malaysian Food Act 1983 (Act 281) & Regulations (2014) (Malaysian Food Act 1983). Hence, the compliance of such honeys to the current standard for human consumption can be doubtful, especially in chronic exposure. Review of the literature reveals very limited and sporadic information pertaining to this aspect in Malaysia.

The increasing demand for authentic and high quality stingless bee honey has rendered significant increase in risks of fraud, in attempts to market the lower quality and/or adulterated honeys as the pure expensive ones. Due to its cheaper cost, mixtures of fructose and glucose are commonly used as adulterants for producing low quality honeys (Zhu *et al.,* 2010), and such a practice may deteriorate its nutritional as well as medicinal benefits.

It has been reported that differences in climate and environmental conditions within the vicinity of its foraging area, processing and storage conditions (Chua et al. 2012) as well as the different species of bees (Chua *et al.,* 2012; Moniruzzaman *et al.,* 2014) and geographical regions (Rao *et al.,* 2016) would result in variations in the quality of honeys. Therefore, having the ability to accurately identify the geographical origin of stingless bee honey products become pertinent for unveiling fraudulent practices as well as promoting improvement of quality control and consumer protection. The association between the multi-elements and geographical origins of the stingless bee honey samples are also remains scarce. To the best of our knowledge, to date, there isn't any study in Malaysia on provenance in stingless honey bee focusing in Johor.

1.3 Aims and Objectives of Study

The aim of the study is to investigated the discriminatory power of inter-element association pattern recognition in stingless bee honeys collected for provenance establishment, acquires forensic significance while the objectives of this study are to:

1. Quantify the concentration of twenty elements, namely Ag, Al, As, B, Ba, Be, Cd, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Pb, Sb, Se, Sn and Zn in pure stingless bee honey obtained from beekeepers at four different geographical locations (five districts) within Johor and the purportedly pure stingless honey purchased from the market using ICP-OES. These samples were used to verify whether they comply with the standard prescription in the Malaysian law (Food Act 1983 (Act 281) & Regulations (2014)).

2. Compare the concentration of stingless bee honey to the minimal risk level from the standard prescribed in the ATSDR for prolonged consumption or chronic oral exposure health effects.

3. Study the efficiency of multi-element analysis and principal component analysis and linear discriminant analysis at discriminating the geographical origins of the pure stingless honey samples and the purportedly pure stingless honey purchased from the market.

1.4 Scope of Study

Samples of pure stingless honey were harvested from four geographical regions *viz* north (Segamat), west (Kota Tinggi), east (Muar and Batu Pahat) and south (Johor Bahru) in Johor, Malaysia. For each district, samples were collected from one honey beekeeper suggested by the Johor Entrepreneur of Stingless Bee Society in the month of May, June and July 2016. Using a validated ICP-OES, multi-element analysis in all the honey samples was attempted. To study the efficiency of multi-element analysis using PCA and LDA at discriminating the geographical origins of the pure stingless bee honey, samples of the purportedly pure stingless honey purchased from the market (Johor: Johor Bahru and Negeri Sembilan: Seremban) were also included.

1.5 Significance of Study

The importance of this study is to quantify the concentration of multi-elements that will be useful for human consumption indicator especially in long-term consumption and also for provenance establishment between the various origins/districts of purely harvested stingless honey. This study will also reveal the similarity or dissimilarity between purely harvested and purportedly pure stingless bee honeys in terms of multi-element compositions. This present study would also pave the way to applications of chemometrics techniques for forensics practical caseworks in Malaysia.

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